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RESEARCH

Widening the Gene Pool of Sexual Tetraploid Bahiagrass: Generation and Reproductive Characterization of a Sexual Synthetic Tetraploid Population

Zilli A.L., C.A. Acuña*, R.R. Schulz, E.A. Brugnoli, V. Guidalevich, C.L. Quarin, and E.J. Martínez

ABSTRACT

The improvement of bahiagrass, *Paspalum notatum* Flüggé, has been inhibited by reduced levels of genetic diversity in sexual tetraploid germplasm. A few experimental sexual tetraploid genotypes (ESTGs) have been generated by chromosome doubling, but these plants typically exhibit low vigor. The objectives of this work were to generate and characterize the ploidy level, mode of reproduction, and fertility of a novel 308 individual sexual synthetic tetraploid population (SSTP) developed by intercrossing 29 sexual F_1 hybrids originated by hybridizing several naturally occurring apomictic tetraploids from diverse origin with a few ESTGs. Ploidy levels were determined using flow cytometry, and reproductive modes were evaluated by a molecular assay with apospory-linked markers and embryo sac observations. The tetraploid level and the sexual mode of reproduction remained stable after two cycles of recombination during the generation of the SSTP. Fertility was evaluated based on seed set under self- and open pollination during 3 yr. The SSTP exhibited in average 30.2 and 15.2% seed set under open and self-pollination, respectively, showing a predominantly cross-pollination behavior with variable levels of self-fertility. There were no differences in terms of fertility between the SSTP and the ESTG. The novel tetraploid population behaves as sexual and cross-pollinated, and it is expected to allow a more efficient genetic improvement under the proposed breeding approaches.

Instituto de Botánica del Nordeste, Consejo Nacional de Investigaciones Científicas y Técnicas, Facultad de Ciencias Agrarias, Univ. Nacional del Nordeste, Sargento Cabral 2131, Corrientes, Argentina. Received 28 July 2017. Accepted 20 Oct. 2017. *Corresponding author (cacuna@agr.unne.edu.ar). Assigned to Associate Editor Joseph Robins.

Abbreviations: ACGR, apospory-controlling genomic region; EDTA, ethylenediaminetetraacetic acid; ESTG, experimental sexual tetraploid genotype; PCR, polymerase chain reaction; SSTP, sexual synthetic tetraploid population.

Warm-season apomictic forage species are widely cultivated around the world and represent an important part of the gross national product of agriculture-based countries (Jank et al., 2014). Most apomictic forages have been released as the result of selection from wild-types (Vogel and Burson, 2004; Miles, 2007) and have been successful because of their superior agronomic characteristics and the possibility of asexual reproduction through seed that remain stable after successive cropping cycles. However, this method of cultivar selection in apomictic species is limited to evaluating and selecting superior genotypes from what is found in nature because of the reproductive barriers between apomictic plants, which are generally associated with polyploidy, and sexual diploids (Vogel and Burson, 2004).

Bahiagrass is a warm-season perennial grass, native to the New World, growing from central eastern Mexico to central Argentina and along the Caribbean Islands (Chase, 1929). It is a primary constituent of grasslands in southern Brazil, Paraguay, Uruguay, and northeastern Argentina. The species was introduced to the southeastern United States and is now one of the most important perennial cultivated forage species (Blount and Acuña, 2009). Bahiagrass has also been introduced to other countries where it is used as turf and forage (Gates et al., 2004). Bahiagrass has two

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different cytotypes: the diploid ($2n = 2x = 20$), which is sexual and cross-pollinated (Burton, 1955); and the tetraploid ($2n = 4x = 40$), which is an aposporous apomictic (Gates et al., 2004). The diploid cytotype is native of a narrow region in northeastern Argentina (Burton, 1967; Daurelio et al., 2004), while the tetraploid cytotype grows across the entire natural range (Burton, 1948).

Experimental sexual tetraploid genotypes have been generated through chromosome doubling (Forbes and Burton, 1961; Quarin et al., 2001; Quesenberry et al., 2010); however, some of these induced plants reproduce by facultative apomixis (Quarin et al., 2001; Acuña et al., 2007). This finding is a clear obstacle for breeding programs because of the possibility of asexual reproduction within potential female parents. In addition, sexual tetraploid plants are difficult to obtain through chromosome duplication treatments and often exhibit low vigor and fertility. All combined, the very narrow gene pool found in the few sexual tetraploid germplasms available limits the possibilities of genetically improving bahiagrass through hybridization.

Aposporous apomixis in bahiagrass is controlled by a single dominant factor that segregates in a distorted pattern that favors the sexual progeny as a result of a pleiotropic lethal effect with incomplete penetrance of the apospory locus (Martínez et al., 2001). Several molecular markers linked to this apospory have been identified in studies conducted to map the apospory-controlling genomic region (ACGR) (Martínez et al., 2003, Stein et al., 2007; Rebozzio et al., 2012). Moreover, comparative molecular analyses and cytological studies revealed that the ACGR in bahiagrass appears to be located in a chromosomal region where genetic recombination is suppressed (Pupilli et al., 2004; Stein et al., 2004; 2007). The presence of chromosome rearrangements with preferential pairing has been proposed as the possible cause of apospory-distorted segregation in bahiagrass (Stein et al., 2004). This hypothesis is supported by the higher frequency of abnormal meiosis present in natural apomictic plants than sexual ones (Dahmer et al., 2008; Podio et al., 2012).

Hybridization between induced sexual tetraploid and apomictic plants has resulted in progeny segregating for different agronomic and reproductive traits (Acuña et al., 2009). The generated sexual hybrids exhibited higher vigor and fertility than the induced sexual plants. Although these sexual hybrids had variable levels of self-fertility, self-compatibility increased in comparison with the induced plants. This could represent an obstacle for breeding programs because of the possible occurrence of inbreeding depression.

Recent studies reported the presence of high genetic diversity among apomictic tetraploid populations of bahiagrass (Daurelio et al., 2004; Cidade et al., 2009; 2010; Reyno et al., 2012). Although this variability is currently locked in apomictic genotypes, it could be released by intraspecific crosses with experimental sexual tetraploids. Furthermore, a sexual synthetic tetraploid population may be generated with the objective of transferring the diversity present in the apomictic gene pool to the sexual germplasm by intercrossing sexual hybrids with different origins. A similar breeding scheme was successfully used on *Brachiaria* spp. grasses by Miles and Escandón (1997). However, in their research interspecific crosses were made using 10 apomictic ecotypes of two different species without taking into consideration genetic diversity because of the lack of sexuality in the same species.

Previously, our research group created 11 families comprised of 524 hybrids by crossing three experimental sexual tetraploids and nine natural apomictic genotypes from different geographic origins and phenotypic characteristics (Zilli et al., 2015). Mode of reproduction in each hybrid had been determined using a molecular marker completely linked to apospory and mature embryo sac observations prior to crossing. It may be possible to obtain a sexual synthetic population of bahiagrass with a broad genetic base by intercrossing verified sexual genotypes from the 11 families generated in previous studies.

The availability of sexual synthetic tetraploid germplasm would allow a simple mass or family selection method, such as recurrent selection by specific combining ability (Hull, 1945), to improve bahiagrass. Or, both specific and general combining ability (Comstock et al., 1949) could be studied through test crosses between selected sexual individuals from the breeding population and elite apomictic genotypes. Early identification and testing of apomictic hybrids for their potential as cultivars is possible because reliable molecular markers completely linked to apospory are available for bahiagrass (Martínez et al., 2003; Zilli et al., 2015).

Acuña et al. (2007) postulated that the female parent in a sexual–apomictic tetraploid bahiagrass cross needs four main characteristics: (i) the same ploidy level, (ii) high vigor, (iii) high sexual expression, and (iv) high cross-fertility. A fifth feature, low self-fertility or high self-incompatibility, would also be desirable because of the possible repercussions of inbreeding depression in a plant breeding program using sexual synthetic germplasm.

The objectives of this research were to (i) generate a SSTP of bahiagrass by intercrossing sexual F_1 hybrids created previously (Zilli et al., 2015), (ii) evaluate the stability of the novel SSTP for ploidy level and mode of reproduction, and (iii) determine the fertility level under self- and cross-pollination of the SSTP in comparison to the few available experimental sexual tetraploid genotypes.

MATERIALS AND METHODS

Plant Material and Crosses

Origin and mode of reproduction of each genotype involved in the generation of the F_1 hybrids used for the present research can be found on Table 1. For this

Table 1. Identification, mode of reproduction and origin of the bahiagrass genotypes used in this work.

Accession	Reproductive mode	Origin
SWSB	Sexual	Sexual White Stigma Bahiagrass, derived from hybrids originally generated by G.W. Burton by crossing an induced tetraploid plant with a apomictic natural tetraploid Bahiagrass with white stigmata, WSB (Burton and Forbes, 1961) and then introduced to Argentina in 1979 (Quarin et al., 1984)
Q4205	Sexual	Obtained by self-pollination of SWSB (Quarin et al., 2003)
C4-4*x*	Sexual	Colchicine-induced tetraploid from a diploid plant collected at Cayastá, Santa Fe, Argentina (Quarin et al., 2001)
CyA1556	Apomictic	Route 35, Km. 364 near Fortín Olavarría, Buenos Aires, Argentina
Q3838	Apomictic	Riachuelo, Corrientes, Argentina
V14327	Apomictic	Capivari, RS, Brazil
Q3775	Apomictic	Tamaulipas, México
Q4064	Apomictic	Saladas, Corrientes, Argentina
SV2893	Apomictic	El Huayo, Department of Cajamarca, province of Cajabamba, Peru
Q3776	Apomictic	Villa Tunari, Chapare´s region, Bolivia
Q4294	Apomictic	Los Algarrobos, Santa Rosa de Calamuchita, Córdoba Argentina
B229	Apomictic	Itaqui, RS, Brazil
TB86	Apomictic	Route 26, Km. 389, Department of Cerro Largo, Uruguay
Argentine	Apomictic	USDA PI 148996. Imported from the United States in 2010 (granted by Dr. Ann Blount, University of Florida)

Table 2. Identity of F_1 families, number of sexual parental F_1 hybrids included in the intercrossing, number of offspring from each sexual F_1 family included in the sexual synthetic tetraploid population (SSTP), and the proportional contribution of each F_1 family to the SSTP.

Crosses						
Female	Male	ID	No. of sexual hybrids in each F_1 family	No. of sexual F_1 included in the intercrossing	No. of offspring on the SSTP	Contribution to SSTP
						%
SWSB	B229	A	37	4	44	14.3
SWSB	SV2893	B	38	3	33	10.7
SWSB	V14327	C	25	3	33	10.7
SWSB	Q3775	D	36	2	22	7.1
SWSB	TB86	E	43	3	32	10.4
Q4205	Q3776	F	42	4	44	14.3
Q4205	CyA1556	G	43	2	15	4.5
Q4205	Q4064	H	35	4	44	14.3
C4-4*x*	Q3838	I	29	1	8	2.6
C4-4*x*	Q4294	J	41	3	33	10.7
Total			369	29	308	100

work, we have used nine F_1 families previously generated by crossing among three experimental sexual tetraploid and nine different apomictic parents (Zilli et al., 2015). Additionally, in the present work we included a 10th F_1 family labeled E (Table 2) that was generated by crossing the sexual genotype SWSB as female parent and a natural apomictic TB86. The progeny from each cross was referred to as a family and a capital letter was given to identify each one (Table 2). Each genotype from the F_1 families was previously identified as hybrid and classified for mode of reproduction (Zilli et al., 2015).

A total of 29 sexual F_1 hybrids, representing the 10 selected families, were intercrossed during the 2013 flowering season (Table 2). The number of sexual F_1 hybrids per families involved in the intercrossing was related to the phenotypic variation observed in each family and the number of plants flowering at the same time (Table 2). A day before anthesis, rhizomes bearing panicles from each parental plant were collected and placed in containers with water. Between 13 and 20 inflorescences from different sexual F_1 hybrids (a single inflorescence per hybrid) were intercrossed on three different occasions depending on the timing of anthesis in these parental plants. All inflorescences were covered together with a glassine bag to prevent contamination with pollen from undesired sources and then placed in a shaded corner of a greenhouse. At the time of anthesis, around sunrise, the bags were shaken to ensure cross-pollination. This procedure was performed for 5 d after the initiation of anthesis. Those inflorescences that had not flowered after the second day were discarded. After

30 d from pollination, inflorescences of each hybrid were manually threshed and seeds were separated using a seed blower. Seeds of each plant were dried in an oven at 37°C for 48 h and then stored individually in paper envelopes.

Seeds of each plant were scarified with 98% sulfuric acid for 10 min, washed, and sown in a greenhouse on potting mix. Seedlings with 3 leaves were transplanted to 150-mL cell seedling flats and after 30 d were planted on 1-m^2 grid field plots located at the Campus of Universidad Nacional del Nordeste, Corrientes, Argentina, in November 2013. The progeny from each half-sib family was randomly planted into the field. One month after planting, 15 g per plant of 15–15–15 (N-P-K) was applied. Plants were cut at the end of each growing season with an 8-cm stubble height.

Mode of Reproduction

Molecular Analysis

Genomic DNA from the apical meristem of each plant was extracted following the method described by Brugnoli et al. (2014). A sample of 50 mg of young leaves were macerated with a plastic fuse drill in 700 μL of extraction buffer cetyl trimethylammonium bromide 2% (100 mM Tris-HCl pH 7.5; 50 mM ethylenediaminetetraacetic acid [EDTA] pH 8; 700 mM NaCl; 140 mM β-mercaptoethanol) in a 1.5-mL tube. The samples were incubated at 65°C for 30 min. After that, 500 μL of chloroform was added and the mixture was shaken for 5 min and then centrifuged for 10 min. Supernatant was recovered and transferred to another clean tube. The DNA was precipitated with 500 μL of cold 2-iso-propanol. The samples were put in a freezer at −20°C for 30 min and then centrifuged at 4°C for 20 min. The aqueous phase was discarded, and the pellet was washed with a washing solution (EtOH 70° + 0.2 M NaOAc) and centrifuged again for 10 min. After centrifugation, the supernatant was discarded, and the DNA was suspended in 50 μL of sterile, tris-ethylenediaminetetraacetic acid buffer (10 mM Tris-HC1 pH 8; 1 mM EDTA pH 8) and kept in a refrigerator. The DNA quantity and integrity was estimated using known concentration DNA patterns, which were separated by electrophoresis in 1% w/v agarose gel containing 1× TAE buffer (40 mM Tris-HCl, 5 mM sodium acetate, 0.77 mM EDTA, pH 8.0) at 40 V for 60 min. The DNA was stained with ethidium bromide (1 mg mL^{-1}) for 30 min, visualized with ultraviolet light, and photographed using a GelDoc-it Imaging System (UVP, LLC).

To ensure that all plants from the synthetic population were sexual, two RAPD markers (UBC243-377 and UBC259-1157) that are completely linked to the apospory locus in bahiagrass were used (Martínez et al., 2003). Polymerase chain reactions (PCRs) were performed according to instructions described by Martínez et al. (2003). The PCR products were separated by electrophoresis in 2% w/v agarose gel in 1× TAE (40 mM Tris-HCl, 5 mM sodium acetate, 0.77 mM EDTA, pH 8.0) at 70 V for 3 h. Gels were stained and visualized as described above. Plants that did not amplify any RAPD marker linked to apospory were classified as sexual. The sexual plants included in the intercross and an apomictic cultivar named Argentine (Table 1) were used as negative and positive controls, respectively.

Cytoembryological Analysis

The 29 parental plants used in the intercrossing, and a sample of 30 plants (10%) from the resulting SSTP were analyzed for mode of reproduction by mature embryo sac observations using the pistils clearing technique described by Zilli et al. (2015). Spikelets at anthesis were fixed in a solution of 70% ethanol, 37% formaldehyde, and glacial acetic acid in the ratio 18:1:1. A minimum of 25 pistils per plant from at least two panicles were observed with a differential interference contrast microscope. Each plant was classified as either sexual or apomictic according to the embryo sac types observed in the ovules. Ovules bearing a single embryo sac with the egg cell, a binucleated central cell, and a mass of antipodal cells were classified as meiotically derived. Ovules bearing single or multiple embryo sacs with an egg cell, a central cell with two nucleus, and no antipodals were classified as aposporic. Ovules containing both aposporous and meiotically derived embryo sacs were classified as mixed. Those ovules with absent or undeveloped embryo sacs were classified as aborted. In turn, plants that showed only meiotically derived or aborted embryo sacs were classified as sexual whereas those showing at least one aposporous embryo sac as apomictic.

Ploidy-Level Analysis

Flow cytometry was used to determine the ploidy level on 10% of the SSTP. Bulks of five plants plus a known tetraploid bahiagrass (Argentine) as control were processed together. Leaves from the plants were collected and ~0.5 cm of length of each was chopped in a Petri dish containing 0.5 mL of nuclei extraction buffer of CyStain UV Precise P (05-5002 from Partec). After 30 s, the material was filtered (50-μm filter) and stained with DAPI (4′, 6-diamidino-2-phenylindole) during 3 min using 1.5 mL of staining buffer CyStain UV Precise P. Samples were processed on a Ploidy Analizer PA II (Partec). A minimum of 3000 nuclei were counted. The rationale for using bulks of five plants was to save time and reduce costs of the analysis. If histograms eventually showed peaks different than expected, the five plants could be reanalyzed and compared one at a time with the control.

Pollination Techniques and Seed Set

Seed set was measured under two treatments: self- and open pollination in January 2014, 2015, and 2016.

Throughout the three growing seasons, between 29 and 31 plants from the SSTP were evaluated, and three ESTGs were added in 2016 for comparisons. Self-pollination seed set was measured after enclosing between three and four panicles per plant in a glassine bag each day before anthesis to force pollination with its own pollen. Open-pollination seed set was measured using three or four panicles per plant, which were bagged after anthesis to avoid seed loss. Inflorescences were manually threshed 30 d after anthesis, and seeds were separated from the empty spikelets with a seed blower. Seed set (Ss) for each plant was calculated with the following formula: Seed set = (No. seeds/total No. spikelets)×100.

Seed Germination

Seeds harvested under open-pollination conditions in 2016 were sown in October 2016 in flats with potting mix under greenhouse conditions. A completely randomized design with two replicas and an experimental unit of 50 seeds was used. Seed germination was calculated 21 d after sowing.

Statistical Analysis

Fertility data were analyzed using Info-Gen software (Balzarini and Di Rienzo, 2013) as a completely randomized design, where each plant represented a replication inside of the population (SSTP, ESTG). Statistical comparisons for seed set were tested in the following order: (i) pollination method comparisons (self- vs. open pollination) of the SSTP during each year, (ii) year comparisons (2014 vs. 2015 vs. 2016) for self- and open pollination on the SSTP, (iii) population comparisons during year 2016 (SSTP vs. ESTG) for each pollination method, and (iv) F_1 and SSTP seed set comparison through grouping plants according to a common female ancestor (Q4205 vs. SWSB vs. C4-4*x*). Germination was compared between SSTP and ESTG. Means, coefficients of variation, and ANOVA were calculated. The Duncan's test was used when more than two means were compared, while LSD was used when two means were compared. Unless otherwise stated in the text, all differences refer to significance at $p < 0.05$. Broad-sense heritability for seed set was estimated through an ANOVA based on 20 genotypes of the SSTP using years of evaluation (2014 and 2015) as replications. Broad-sense heritability for seed germination was also estimated through an ANOVA.

RESULTS

Generation and Ploidy Level of the Sexual Synthetic Tetraploid Population

A sexual synthetic tetraploid bahiagrass population (SSTP) of 308 plants was generated by recombining 29 sexual hybrids obtained from the F_1 families developed by Zilli et al. (2015) through a controlled intercrossing. Eleven

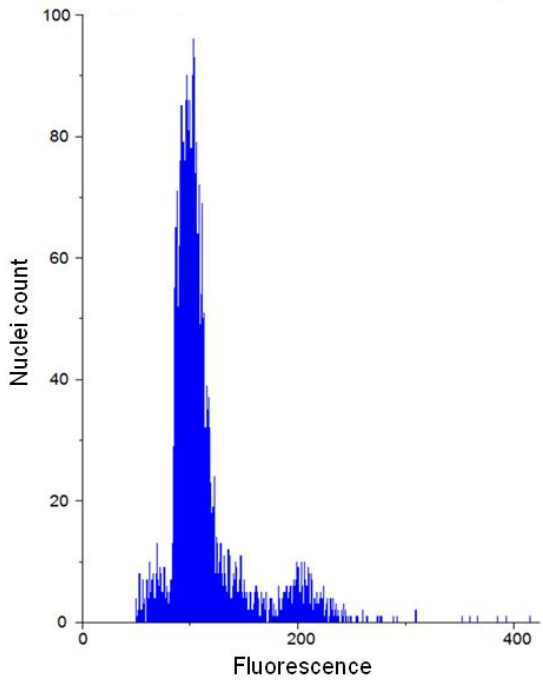

Fig. 1. Flow cytometry histogram generated with a bulk of five samples from the sexual synthetic tetraploid population plus the tetraploid check. The higher peak corresponds to nucleus in phase G1 (CV = 10.79) and the lower peak to nucleus in phase G2 (CV = 8.52) from tetraploid plants ($2n = 4x = 40$).

descendants per sexual parental plant were planted into the field, with the exception of F_1 hybrids A29, B19, and C11 in which four, eight, and 10 offspring were planted, respectively. The number of sexual F_1 hybrids from each family involved in the intercrossing, and the proportional contribution to the SSTP are shown on Table 2.

All plants sampled from the SSTP were tetraploid ($2n = 4x = 40$) as determined by flow cytometry (Fig. 1). These results indicate that ploidy level of the population has remained constant after two recombination cycles.

Mode of Reproduction

Mode of reproduction was determined based on molecular markers in the 308 plants from the SSTP. Two RAPD markers that are 100% linked to apospory were not amplified in the 306 genotypes; therefore, they were classified as sexual (Fig. 2). Amplification of both apospory linked markers was observed in the remaining two plants of the SSTP, so they were classified as apomictic.

Embryo sac observations were performed on 28 randomly selected plants from the SSTP and the two plants preliminarily classified as apomictic by the RAPD markers. Only meiotic and aborted embryo sacs were found in the 28 randomly selected plants (Fig. 3a), therefore, they were confirmed to be sexual. The proportion of meiotic embryo sacs varied between 46.2 and 97.1%, with a mean of 83.4% (Fig. 4). A range of 2.9 to 53.8% of aborted embryo sacs, with a mean of 16.6%, was also

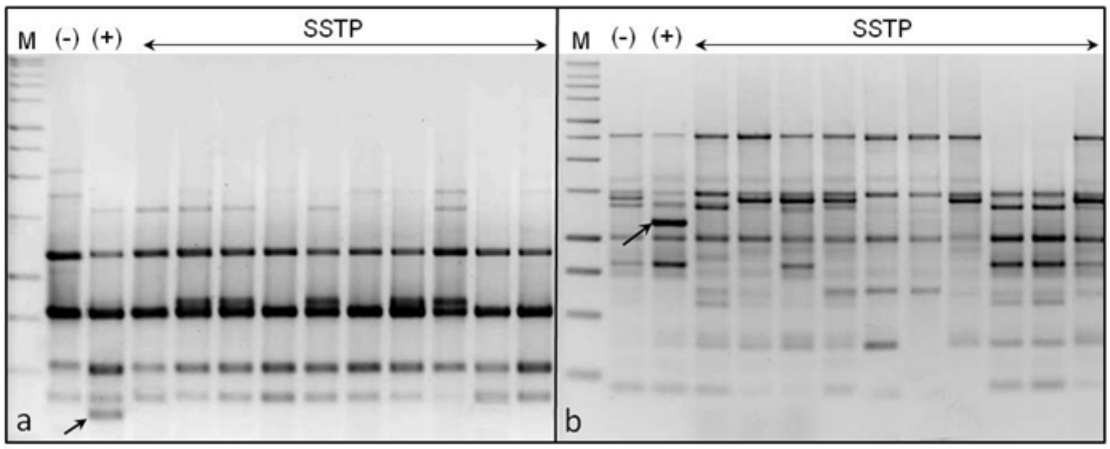

Fig. 2. Random amplified polymorphic DNA markers profiles: (a) generated using the UBC243 primer on 10 plants of the sexual synthetic tetraploid population (SSTP). Arrow indicates the apospory-linked molecular marker (377 bp); (b) generated using the UBC259 primer on 10 plants of the SSTP. Arrow indicates the apospory-linked molecular marker (1157 bp). M, 1 kb molecular marker; (–), sexual check; (+), apomictic check.

observed among genotypes (Fig. 4). Plants C13#8 and F29#3, preliminarily classified as apomictic, exhibited 68% ovules bearing aposporous embryo sacs, therefore, their apomictic behavior was confirmed (Fig. 3b, 4). The fraction of meiotic (3.8%) and aborted (28.3%) embryo sacs was similar between both genotypes (Fig. 4).

Seed Fertility

The mean seed set for the SSTP under open-pollinating conditions was 23.4, 39.3, and 28.0% in 2014, 2015, and 2016, respectively. Seed set under self-pollinating conditions was 8.5, 20.5, and 16.6% during 2014, 2015, and 2016, respectively (Table 3). Open-pollination fertility was significantly higher than in self-pollination ($p < 0.001$) during all 3 yr indicating that the SSTP behaves as

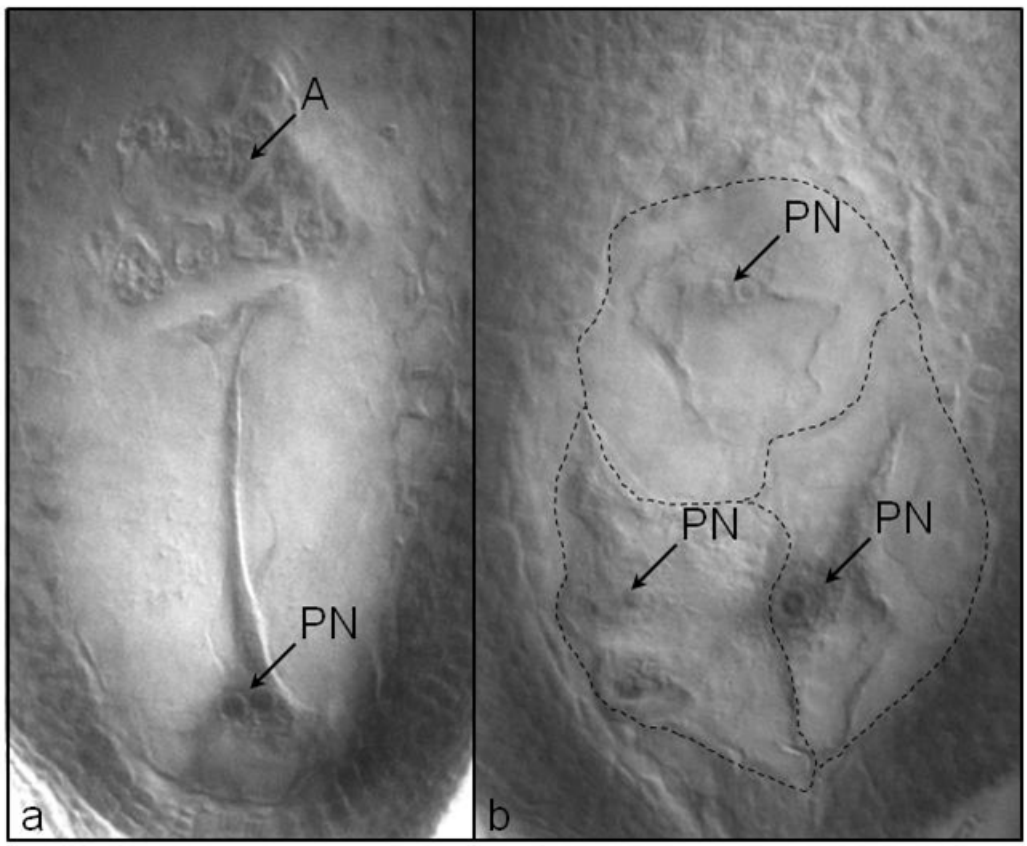

Fig. 3. Embryo sacs of the sexual synthetic tetraploid population of bahiagrass: (a) A meiotically derived embryo sac containing the antipodes (A) and the polar nuclei (PN); (b) A group of three aposporous embryo sacs within the same ovule.

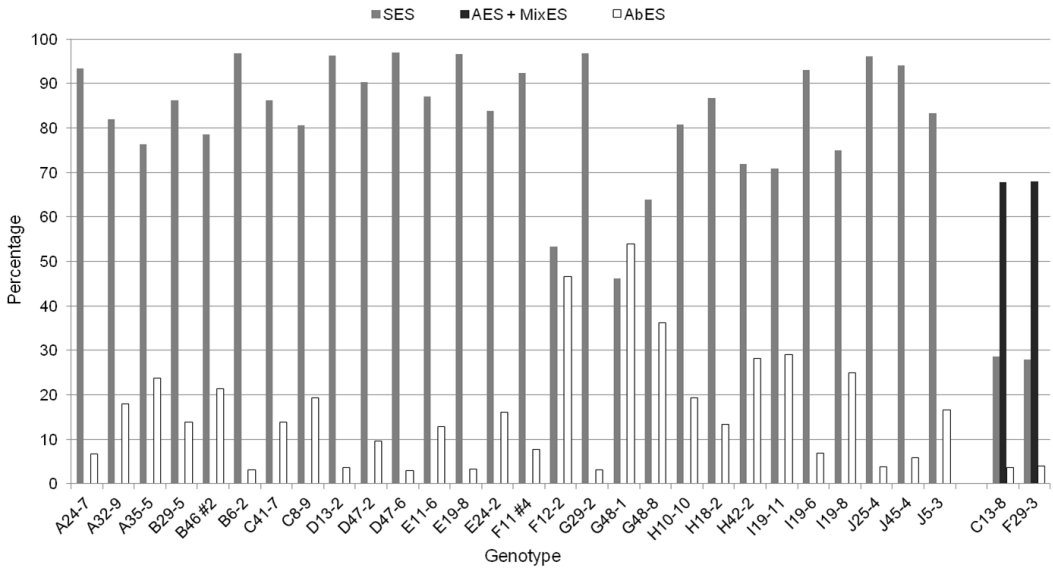

Fig. 4. Proportion of embryo sacs for each analyzed genotype from the sexual synthetic tetraploid population. SES, sexual embryo sacs; AES+MixES, aposporous embryo sacs plus mixed embryo sacs (SES + AES); AbES, aborted embryo sacs.

a cross-pollinated population with variable levels of self-fertility. A significant correlation between seed set under self- and open pollination was found across years ($r = 0.69$; $p < 0.001$).

Year of evaluation was a significant effect in both self-pollinated ($p < 0.001$) and open-pollinated conditions ($p < 0.001$) according to the analysis of variance. Seed set for self-pollination was significantly lower in 2014 than for 2015 and 2016, whereas in open pollination, seed set was significantly higher during 2015 than that observed in 2014 and 2016.

The ANOVA showed that seed set for both self- and open pollination was highly variable depending on the genotype and the year of evaluation ($p < 0.001$). This high variability was represented by the coefficient of variation, which in open pollination was 66.9, 31.2, and 60.7% for 2014, 2015, and 2016 respectively, with an overall average CV of 57.9%. Also, the CV in self-pollination was 83.3, 61.1, and 65.4% for 2014, 2015, and 2016 respectively, with an overall average of 75.0% (Table 3). Heritability for seed set was 0.59 and 0.44 for self- and open pollination, respectively.

The three ESTG reached a mean of 9.9 and 22.5% seed set under self- and open pollination, respectively (Table 3). The coefficients of variation for these means were high (58.0 and 41.1% for self- and open pollination, respectively) mainly because of differences between the induced sexual tetraploid C4-4*x* (3.4 and 12.3% of seed set under self- and open pollination, respectively) and the genotypes generated by hybridization SWSB and Q4205 (13.2 and 27.6% of seed set under self- and open pollination, respectively). No significant differences were observed between SSTP and ESTG for both self- and open pollination during 2016 ($p = 0.58$ and 0.13, respectively).

When the 29 sexual F_1 hybrids were grouped according to a common female ancestor (SWSB, Q4205, and C4-4*x*),

Table 3. Seed set observed in the different sexual populations, years of evaluation, and pollination method.

Population†	Year	Pollination method	No. of plants	Seed set			
				Mean	Minimum	Maximum	CV
				%			
F_1SH	2013	Intercross	29	50.28	5.00	81.00	36.20
SSTP	2014	Self	29	8.47	0.00	25.15	83.31
		Open	29	23.35	4.73	60.88	66.87
SSTP	2015	Self	31	20.54	2.56	63.48	61.11
		Open	31	39.34	13.42	69.04	31.21
SSTP	2016	Self	31	16.64	0.00	39.18	65.42
		Open	31	28.01	2.83	57.96	60.70
ESTG	2016	Self	3	9.90	3.40	14.30	58.03
		Open	3	22.47	12.30	30.30	41.06

† F_1SH, F_1 sexual hybrids; SSTP, sexual synthetic tetraploid population; ESTG, experimental sexual tetraploid genotypes.

no significant differences were observed for seed set (p = 0.96). In addition, when individuals from the SSTP were grouped according to the same female ancestor, no significant differences were observed between the three groups for both self- and open pollination on the three analyzed periods ($p > 0.25$).

Seed germination in the SSTP was 51.1% (CV = 43.2) and significantly higher than the 26% (CV = 121) obtained for ESTG. Heritability for seed germination was 0.79.

DISCUSSION

Genetic improvement in apomictic forage species by mean of hybridization requires sexual germplasm at the same ploidy level of the apomictic one (Acuña et al., 2009). In addition, this sexual germplasm should contain the maximum amount of genetic variability as possible to increase the likelihood of success in the breeding efforts (Poehlman and Sleper, 1995). A novel breeding approach was used in our research attempting to transfer the inherent diversity present in apomictic bahiagrass germplasm to a sexual synthetic tetraploid population with the objective of increasing the available variability in sexual bahiagrass germplasm. The ploidy level, mode of reproduction, and fertility of the novel SSTP was evaluated considering its potential value for breeding this species.

The SSTP was developed in the following three phases. First, 10 F_1 families were generated from crosses between three ESTG and 10 naturally occurring apomictic genotypes to transfer the genetic diversity available in this apomictic germplasm to the new hybrids. Second, these hybrids were classified for mode of reproduction using molecular and cytological methods. Finally, a group of sexual hybrids were selected and intercrossed to generate the SSTP. The contribution of each F_1 family to the SSTP differed and was related to the phenotypic variability present in each. A similar breeding scheme was useful to develop a vigorous sexual synthetic *Brachiaria* population resistant to three species of spittlebug in Colombia after six cycles of recurrent selection (Miles and do Valle, 1996; Miles and Escandón, 1997; Miles et al., 2004; 2006).

The use of two RAPD markers that are 100% linked to the apospory locus in bahiagrass (Martínez et al., 2003) allowed a quick confirmation of the mode of reproduction in all the SSTP genotypes during early stages of plant development. A random cytoembriological analysis of 10% of the SSTP based on mature embryo sac observations corroborated the molecular results. There are advantages and disadvantages when analyzing the mode of reproduction with either of these techniques. On one hand, molecular markers allow an early classification of plants, even at the seedling stage, which is very useful for breeding purposes. Nevertheless, markers do not provide information regarding the expression level of apospory, which is necessary in case of using this method for apomictic hybrid selection. Cytoembriological analysis by mature embryo sac observations does provide data about expression of apospory in apomictic plants; however, this technique demands more time and requires flowering plants. Although the linked markers provide sufficient information for separating the sexual germplasm, both methods (molecular and cytological) are needed to identify highly apomictic hybrids.

The stability of sexuality over successive generations is very important for breeding tetraploid germplasm using the novel sexual population. The mode of reproduction of the 29 sexual F_1 hybrids used in the intercrossing had been determined previously with a RAPD marker completely linked to apospory in bahiagrass (Zilli et al., 2015). In the present work, we corroborated by cytoembriological analysis that the 29 individuals reproduced sexually. Taking into consideration that a single dominant Mendelian factor controls apospory in bahiagrass, where sexuality behaves as recessive (aaaa) (Martínez et al., 2001), no apomictic offspring should be expected in crosses between sexual genotypes. The identification of two apomictic plants in the SSTP may be the result of contamination with pollen from an undesired source during intercrossing. These findings permitted us to discard the two apomictic genotypes and maintain the purity of the population. Independent of the reason for the presence of two apomictic plants within the SSTP, 99.4% of the progeny was sexual, indicating that sexuality is expected to be highly stable across selection cycles within the SSTP, but that complete isolation from tetraploid apomictic pollen sources is paramount.

Seed set under open pollination in the SSTP averaged 30.2% for the three evaluated years. This level was similar to the 26% found by Burton et al. (1970) for induced sexual tetraploid genotypes and higher than the 14% reported by Acuña et al. (2007) in genotypes of the same origin. Acuña et al. (2009; 2011) reported 41 and 30% seed set for the first and second generation of sexual tetraploid hybrids (sexual × apomictic crosses) under open and cross-pollination, respectively. These two values were similar to those observed in this study for the SSTP. Burton (1946) evaluated seed set under open pollination of different bahiagrass types, finding an overall of 75.9% for the natural sexual diploid 'Pensacola', and 62.7% for naturally occurring apomictic tetraploids. Both values are higher than those reported for induced sexual tetraploid genotypes and sexual tetraploid genotypes originating from hybridization as reported herein. However, the seed set values observed in this study were similar to those reported for other sexual warm-season perennial grasses such as ruzigrass (*Brachiaria ruziziensis* R. Germ. & C. M. Evrard) (22%), African bristle grass [*Setaria sphacelata* (Schumach.) Stapf & C. E. Hubb.] (43%) (Parihar and Pathak, 2006), diploid (37%) and tetraploid (30%) Rhodes grass (*Chloris gayana* Kunth) (Kokubu and Taira, 1982). Therefore, the level of seed set under open pollination obtained in the SSTP will not be an obstacle

for its use in breeding programs. Additionally, the high variability found for this trait in the SSTP will allow the selection of genotypes with high levels of cross-fertility to be included during cultivar development in the future.

Self-fertility was highly variable in the SSTP, with an overall mean of 15.2% during the three evaluated years. These results are different from those reported by Burton et al. (1970) and Acuña et al. (2007), where the researchers observed self-fertility of <0.1 and 2%, respectively. However, the sexual tetraploid genotypes evaluated in both studies were obtained by chromosome doubling with colchicine, which were characterized by low vigor and fertility (Acuña et al., 2007). More recently, Acuña et al. (2009) evaluated self-fertility on (sexual × apomictic) sexual tetraploid F_1 hybrids of bahiagrass over 2 yr and reporting 31% seed set under self-pollination, which is considerably higher than found in both this and other research on induced sexual tetraploid genotypes. Burton (1955) and Acuña et al. (2009; 2011) reported inbreeding depression in progeny generated by self-pollination in diploid and tetraploid hybrids genotypes, respectively. Considering the reported cases of inbreeding depression in sexual bahiagrass germplasm, it would be necessary to identify and select highly self-sterile genotypes of the SSTP for breeding purposes. Variable levels of self-compatibility, similar to those observed in this research, have also been reported in many other cross-pollinated species. For instance, Crowe (1971) studied a population of borage (*Borago officinalis* L.) and found that self-fertility decreased as the level of inbreeding increased. Lundqvist (1958) reported similar results during work on diploid and tetraploid populations of rye (*Secale cereale* L.) that demonstrated heterozygosity at the S-Z loci resulted in a more self-fertile plant. Numerous alleles have been reported at self-compatibility loci in other grass species (Trang et al., 1982; Devey et al., 1994; Fearon et al., 1994). It is expected that several different alleles were incorporated into the SSTP in light of the different origins of the parental material. High levels of heterozygosity, and a corresponding increase in self-fertility, should be expected in this population after two cycles of recombination. Moreover, accelerated inbreeding resulting from the induction of autotetraploidy in bahiagrass could also explain the reports of low self-fertility (Burton et al., 1970; Acuña et al., 2007), as well as with the colchicine-induced tetraploid genotype C4-4*x* used in this research, which exhibited 3.4% seed set when self-pollinated.

Variable environmental conditions were likely responsible for the significantly different trends in seed set for the SSTP under both pollination methods during each year (Table 3). Similarly, years of evaluation significantly impacted seed set in both self- and open-pollinated induced sexual tetraploid genotypes of bahiagrass (Acuña et al., 2007). Therefore, seed set should be determined in more than 1 yr to obtain representative results.

Experimental sexual tetraploid genotypes showed two-fold higher seed set under open pollination (22.5%) than self-pollination (9.9%), indicating that these genotypes behave mainly as out crossers, but with considerable level of self-fertility. The genotype C4-4*x* exhibited similar fertility to the previously reported values for induced sexual tetraploids (Burton et al., 1970; Acuña et al., 2007). Genotypes originating from crosses between the female parents SWSB or Q4205 with apomictic tetraploid genotypes showed similar fertility to those reported by Acuña (2006) for genotypes of the same origin. The higher degree of fertility found in sexual genotypes derived from crosses indicates that fertility of induced sexual genotypes can be restored, at least in part, by hybridization. Fertility comparisons between the different populations during 2016 determined that the SSTP reached the same fertility level observed in the ESTG, which represents current sexual tetraploid germplasm of the species. However, the SSTP has the advantage of containing a wider gene pool that was transferred from highly heterozygotic natural apomictic genotypes through two cycles of recombination. In addition, the SSTP showed an improvement on seed germination compared with ESTG. The high variability and heritability observed for this trait in the new germplasm would be useful for breeding the species.

In lieu of the reported cases of heterosis in bahiagrass (Acuña et al., 2011; Zilli et al., 2015), a recurrent selection scheme targeting superior combining ability (Hull, 1945; Comstock et al., 1949) could be appropriate for improving this sexual population because the fact that additive and nonadditive effects could be accumulated through selection cycles. The advantage of this breeding process is that apomictic hybrids generated during test crossing could be evaluated in the field as potential new cultivars (Miles, 2007). The selection of apomictic hybrids in bahiagrass would be straightforward because of the availability of molecular markers linked to apospory, which allows an early detection of the mode of reproduction, thereby saving resources, as only apomictic hybrids would be further studied in the field. A posterior cytoembriological analysis by mature embryo sac observation during flowering would then allow for only the selection of hybrids with high levels of apospory expressivity.

Another suitable breeding scheme for the SSTP is the phenotypic recurrent selection method developed by Burton (1982) for sexual diploid bahiagrass germplasm. This process has been useful for the improvement of dry matter yield, increased germinative energy, and extension of the growing season through by selection against photoperiod sensitivity. The success of this plant breeding improvement procedure is demonstrated by the generation of several improved populations and cultivars such as Tifton 9, Tifton 18, Tifton 23, TifQuik, and UF-Riata (Blount and Acuña, 2009). A similar scheme could be adopted to generate an improved sexual tetraploid population for its release to the

market as an alternative to the release of apomictic cultivars. Moreover, both recurrent phenotypic selection and recurrent selection based on combining ability could be performed together and complement each other.

An analysis of genetic variability in the SSTP is still necessary to evaluate how efficient the transfer of the apomictic gene pool to the sexual one has been in addition to estimating the usefulness this new variability will be for genetic improvement. Considering that seed quality is one of the most limiting factors for bahiagrass adoption for pastures (West and Marousky, 1989), the SSTP could be appropriate as a base population for breeding higher speed of germination and quicker field establishment.

In conclusion, we generated a SSTP with a wide genetic background transferred from naturally occurring apomictic bahiagrass genotypes collected along the natural distribution area of the species. Sexual mode of reproduction and tetraploid level were validated in this population. Comparison of seed set level under open pollination vs. self-pollination indicates that the individuals of SSTP behave mainly as out crossers with variable levels of self-fertility. This information will be useful for planning breeding programs to address weaknesses in the species.

Conflict of Interest Disclosure

The authors declare that there is no conflict of interest.

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References

Acuña, C.A. 2006. Bahiagrass germplasm reproductive characterization, and breeding at the tetraploid level. M.S. thesis. University of Florida, Gainsville.

Acuña, C.A., A.R. Blount, K.H. Quesenberry, W.W. Hanna, and K.E. Kenworthy. 2007. Reproductive Characterization of Bahiagrass Germplasm. Crop Sci. 47:1711–1717. doi:10.2135/cropsci2006.08.0544

Acuña, C.A., A.R. Blount, K.H. Quesenberry, K.E. Kenworthy, and W.W. Hanna. 2009. Bahiagrass tetraploid germplasm: Reproductive and agronomic characterization of segregating progeny. Crop Sci. 49:581–588. doi:10.2135/cropsci2008.07.0402

Acuña, C.A., A.R. Blount, K.H. Quesenberry, K.E. Kenworthy, and W.W. Hanna. 2011. Tetraploid bahiagrass hybrids: Breeding technique, genetic variability and proportion of heterotic hybrids. Euphytica 179:227–235.

Balzarini, M.G., and J.A. Di Rienzo. 2013. InfoGen versión 2013. FCA, Universidad Nacional de Córdoba, Argentina. http://www.info-gen.com.ar

Blount, A.R., and C.A. Acuña. 2009. Bahiagrass. In: R.J. Singh, editor, Genetic resources, chromosome engineering, and crop improvement series: Forage crops. Vol. 5. CRC Press, Boca Raton, FL. p. 81–101. doi:10.1201/9781420047400.ch4

Brugnoli, E.A., M.H. Urbani, C.L. Quarin, A.L. Zilli, E.J. Martínez, and C.A. Acuña. 2014. Diversity in apomictic populations of *Paspalum simplex* Morong. Crop Sci. 54:1656–1664. doi:10.2135/cropsci2013.11.0780

Burton, G.W. 1946. Bahiagrass types. J. Am. Soc. Agron. 38:273–281. doi:10.2134/agronj1946.00021962003800030008x

Burton, G.W. 1948. The method of reproduction in common bahiagrass, *Paspalum notatum*. J. Am. Soc. Agron. 40:443–452. doi:10.2134/agronj1948.00021962004000050008x

Burton, G.W. 1955. Breeding Pensacola bahiagrass, *Paspalum notatum*: Method of reproduction. Agron. J. 47:311–314. doi:10.2134/agronj1955.00021962004700070008x

Burton, G.W. 1967. A search for the origin of Pensacola bahiagrass. Econ. Bot. 21:379–382. doi:10.1007/BF02863165

Burton, G.W. 1982. Recurrent restricted phenotypic selection increases bahiagrass forage yield. Crop Sci. 22:109–111. doi:10.2135/cropsci1982.0011183X002200010025x

Burton, G.W., and I. Forbes. 1961. The genetics and manipulation of obligate apomixis in common bahiagrass (*Paspalum notatum* Flügge). In: C.L. Skidmore, et al., editors, Proc. 8th Internatl. Grassl. Congr. Reading, England. 11–21 July 1960. Alden Press, Oxford, England. p. 66–71.

Burton, G.W., Jr., I. Forbes, and J. Jackson. 1970. Effect of ploidy on fertility and heterosis in Pensacola Bahiagrass. Crop Sci. 10:63–66. doi:10.2135/cropsci1970.0011183X001000010025x

Chase, A. 1929. The North American species of *Paspalum*. Control US Natl. Herb. 28:1–310.

Cidade, F.W., T.T. de Souza-Chies, L.A.R. Batista, M. Dall'Agnol, M.I. Zucchi, L. Jungmann, and A.P. Souza. 2009. Isolation and characterization of microsatellite loci in *Paspalum notatum* Flüggé (Poaceae). Conserv. Genet. 10:1977–1980. doi:10.1007/s10592-009-9872-6

Cidade, F.W., T.T. de Souza-Chies, F.H.D. Souza, L.A.R. Batista, M. Dall'Agnol, J.F.M. Valls, M.I. Zucchi, and A.P. Souza. 2010. Microsatellite loci for *Paspalum atratum* (Poaceae) and cross-amplification in other species. Am. J. Bot. 97:e107–e110. doi:10.3732/ajb.1000207

Comstock, R.E., H.F. Robinson, and P.H. Harvey. 1949. A breeding procedure designed to make maximum use of both general and specific combining ability. Agron. J. 41:360–367. doi:10.2134/agronj1949.00021962004100080006x

Crowe, L.K. 1971. The polygenic control of outbreeding in *Borago officinalis*. Heredity 27:111–118. doi:10.1038/hdy.1971.75

Dahmer, N., M.T. Schifino-Wittmann, M. Dall'Agnol, and B. de Castro. 2008. Cytogenetic data for *Paspalum notatum* Flügge accessions. Sci. Agric. 4:381–388. doi:10.1590/S0103-90162008000400009

Daurelio, L.D., F. Espinoza, C.L. Quarin, and S.C. Pessino. 2004. Genetic diversity in sexual diploid and apomictic tetraploid populations of *Paspalum notatum* situated in sympatry or allopatry. Plant Syst. Evol. 244:189–199. doi:10.1007/s00606-003-0070-6

Devey, F., C.H. Fearon, M.D. Haywardt, and M.J. Lawrence. 1994. Self-incompatibility in ryegrass. XI. Number and

frequency of alleles in a cultivar of *Lolium perenne* L. Heredity 73:262–264. doi:10.1038/hdy.1994.132

Fearon, C.H., M.A. Cornish, M.D. Haywardt, and M.J. Lawrence. 1994. Self-incompatibility in ryegrass. X. Number and frequency of alleles in a natural population of *Lolium perenne* L. Heredity 73:254–261. doi:10.1038/hdy.1994.131

Forbes, I., and G.W. Burton. 1961. Induction of tetraploidy and a rapid field method of detecting induced tetraploidy in Pensacola Bahiagrass. Crop Sci. 1:383–384. doi:10.2135/cropsci1961.0011183X000100050032x

Gates, R.N., C.L. Quarin, and C.G.S. Pedreira. 2004. Bahiagrass. In: L.E. Moser, et al., editors, Warm-season (C4) grasses. Agron. Monogr. 45. ASA, CSSA, SSSA, Madison, WI. p. 651–680.

Hull, F.H. 1945. Recurrent selection for specific combining ability in corn. J. Am. Soc. Agron. 37:134–145. doi:10.2134/agronj1945.00021962003700020006x

Jank, L., S.C. Barrios, C.B. do Valle, R.M. Simeão, and G.F. Alves. 2014. The value of improved pastures to Brazilian beef production. Crop Pasture Sci. 65:1132–1137. doi:10.1071/CP13319

Kokubu, T., and M. Taira. 1982. Some morphological characters and the reproductive method of diploid and tetraploid varieties in rhodes grass, *Chloris gayana* Kunth. Mem. Fac. Agr. Kagoshima Univ. 18:61–68.

Lundqvist, A. 1958. Self-incompatibility in Rye. IV. Factors related to self seeding. Hereditas 44:193–256. doi:10.1111/j.1601-5223.1958.tb03481.x

Martínez, E.J., M.H. Urbani, C.L. Quarin, and J.P.A. Ortiz. 2001. Inheritance of apospory in bahiagrass, *Paspalum notatum*. Hereditas 135:19–25. doi:10.1111/j.1601-5223.2001.00019.x

Martínez, E.J., H.E. Hopp, J. Stein, J.P.A. Ortiz, and C.L. Quarin. 2003. Genetic characterization of apospory in tetraploid *Paspalum notatum* based on the identification of linked molecular markers. Mol. Breed. 12:319–327. doi:10.1023/B:MOLB.0000006868.12568.32

Miles, J.W. 2007. Apomixis for cultivar development in tropical forage grasses. Crop Sci. 47:238–249. doi:10.2135/cropsci2007.04.0016IPBS

Miles, J.W., and C.B. do Valle. 1996. Manipulation of apomixis in *Brachiaria* breeding. In: J.W. Miles, et al., editors, *Brachiaria*: Biology, agronomy, and improvement. CIAT, Cali, Colombia, and CNPGC/EMBRAPA, Campo Grande, MS. p. 164–177.

Miles, J., and M.L. Escandón. 1997. Further evidence on the inheritance of reproductive mode in *Brachiaria*. Can. J. Plant Sci. 77:105–107. doi:10.4141/P95-187

Miles, J.W., C.B. do Valle, I.M. Rao, and V.P.B. Euclides. 2004. Brachiariagrasses. In: L.E. Moser, et al., editors, Warm-season (C4) grasses. Agron. Monogr. 45. ASA, CSSA, SSSA, Madison, WI. p. 745–783.

Miles, J.W., C. Cardona, and G. Sotelo. 2006. Recurrent selection in a synthetic Brachiariagrass population improves resistance to three spittlebug species. Crop Sci. 46:1088–1093. doi:10.2135/cropsci2005.06-0101

Parihar, S.S., and P.S. Pathak. 2006. Flowering phenology and seed biology of selected tropical perennial grasses. Trop. Ecol. 47:81–87.

Poehlman, J.M., and D.A. Sleper. 1995. Breeding field crops. Iowa State Univ. Press, Ames, IA.

Podio, M., L.A. Siena, D. Hojsgaard, J. Stein, C.L. Quarin, and J.P.A. Ortiz. 2012. Evaluation of meiotic abnormalities and pollen viability in aposporous and sexual tetraploid *Paspalum notatum* (Poaceae). Plant Syst. Evol. 298:1625–1633. doi:10.1007/s00606-012-0664-y

Pupilli, F., E.J. Martínez, A. Busti, O. Calderini, C.L. Quarin, and S. Arcioni. 2004. Comparative mapping reveals partial conservation of synteny at the apomixis locus in *Paspalum* spp. Mol. Gen. Genomics 270:539–548. doi:10.1007/s00438-003-0949-5

Quarin, C.L., B.L. Burson, and G.W. Burton. 1984. Cytology of intra- and interspecific hybrids between two cytotypes of *Paspalum notatum* and *P. cromyorrhizon*. Bot. Gaz. 145:420–426. doi:10.1086/337474

Quarin, C.L., F. Espinoza, E.J. Martínez, S.C. Pessino, and O.A. Bovo. 2001. A rise of ploidy level induces the expression of apomixis in *Paspalum notatum*. Sex. Plant Reprod. 13:243–249. doi:10.1007/s004970100070

Quarin, C.L., M.H. Urbani, A.R. Blount, E.J. Martínez, C.M. Hack, G.W. Burton, and K.H. Quesenberry. 2003. Registration of Q4188 and Q4205, sexual tetraploid germplasm lines of Bahiagrass. Crop Sci. 43:745–746. doi:10.2135/cropsci2003.0745

Quesenberry, K.H., J.M. Dampier, Y.Y. Lee, R.L. Smith, and C.A. Acuña. 2010. Doubling the chromosome number of bahiagrass via tissue culture. Euphytica 175:43–50. doi:10.1007/s10681-010-0165-4

Rebozzio, R.N., M.P. Rodríguez, J. Stein, J.P.A. Ortiz, C.L. Quarin, and F. Espinoza. 2012. Validation of molecular markers linked to apospory in tetraploid races of bahiagrass, *Paspalum notatum* Flügge. Mol. Breed. 29:189–198. doi:10.1007/s11032-010-9537-7

Reyno, R., R. Narancio, P. Speranza, J. Do Canto, B. López Carro, P. Hernández, J. Burgueño, D. Real, and M. Dalla Rizza. 2012. Molecular and cytogenetic characterization of a collection of bahiagrass (*Paspalum notatum* Flügge) native to Uruguay. Genet. Resour. Crop Evol. 59:1823–1832. doi:10.1007/s10722-012-9806-x

Stein, J., C.L. Quarin, E.J. Martínez, S.C. Pessino, and J.P.A. Ortiz. 2004. Tetraploid races of *Paspalum notatum* show polysomic inheritance and preferential chromosome pairing around the apospory-controlling locus. Theor. Appl. Genet. 109:186–191. doi:10.1007/s00122-004-1614-z

Stein, J., S.C. Pessino, E.J. Martínez, M.P. Rodriguez, L.A. Siena, C.L. Quarin, and J.P.A. Ortiz. 2007. A genetic map of tetraploid *Paspalum notatum* Flüggé (bahiagrass) based on single-dose molecular markers. Mol. Breed. 20:153–166. doi:10.1007/s11032-007-9083-0

Trang, Q.S., G. Wricke, and W.E. Weber. 1982. Number of alleles at the incompatibility loci in *Secale cereale* L. Theor. Appl. Genet. 63:245–248. doi:10.1007/BF00304003

Vogel, K.P., and B.L. Burson. 2004. Breeding and genetics. In: L.E. Moser, et al., editors, Warm-season (C4) grasses. Agron. Monogr. 45. ASA, CSSA, SSSA, Madison, WI. p. 51–94.

West, S.H., and F.J. Marousky. 1989. Mechanism of dormancy in Pensacola bahiagrass. Crop Sci. 29:787–791. doi:10.2135/cropsci1989.0011183X002900030050x

Zilli, A.L., E.A. Brugnoli, F. Marcón, M.B. Billa, E.F. Rios, E.J. Martínez, and C.A. Acuña. 2015. Heterosis and expressivity of apospory in tetraploid Bahiagrass hybrids. Crop Sci. 55:1189–1201. doi:10.2135/cropsci2014.10.0685